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In the past 12 months we investigated the ability of IKK to regulate β-catenin signaling activity. We found that IKK exists in a complex with β-catenin and that expression of either IKKα or IKKβ can decrease β-catenin signaling. However, only a dominant negative (DN) IKKα mutant increased β-catenin signaling as well as protein levels. In addition, DN IKKα, but not DN IKKβ, inhibited the ability of APC to decrease β-catenin signaling. These results suggest that the IKK proteins are involved in β-catenin regulation.

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Introduction

β-catenin is an oncogene involved in signaling by the wnt pathway. Upon wnt signaling, β-catenin accumulates in the cytoplasm and nucleus where it interacts with Tcf/LEF transcription factors. This complex then activates transcription of several genes, including cyclin D1 and c-myc, indicating that this pathway may be involved in cell cycle regulation (12;19;30;37;41). In the absence of wnt signaling, cytoplasmic β-catenin associates with a complex containing the tumor supressor gene adenomatous polyposis coli (APC), axin, and GSK3β (27-29;32). The activity of this complex results in phosphorylation of β-catenin at N-terminal serine and threonine residues. Mutations in this N-terminal region or mutations of the APC gene lead to accumulation of β-catenin and are associated with many different cancers, including melanoma and colon cancer (reviewed in (26)). Once phosphorylated β-catenin is recognized by F box/WD 40 repeat containing proteins, targeting it for ubiquitination by the SCF E3 ubiquitin ligase and subsequent proteasomal degradation (8;11;18;43). Although GSK-3β is clearly involved in the wnt-mediated regulation of β-catenin stability, several studies indicate that other kinases may also be involved in regulation of β-catenin signaling. For example, both typical and atypical PKCs have been implicated in β-catenin regulation (5;29). However, LiCl, an inhibitor of GSK3β, does not significantly alter the ability of APC to down-regulate β-catenin activity (7). In addition, the GSK3β knockout mouse does not exhibit any changes in β-catenin levels and is morphologically normal up to 12 days of gestation(13). Remarkably, GSK3β knockout mice are characterized by defects similar to those of mice lacking components of the NFκB pathway.

There are several other connections between β-catenin regulation and regulation of IκBα, which inhibits NF-κB by sequestering it in the cytoplasm. The IκBα kinase complex, IKK, regulates NF-κB activation by phosphorylating its inhibitors IκBα, IκBβ, and IκBε on two closely spaced serine residues and targeting them for ubiquitination using the same F box/SCF machinery used for ubiquitination of β-catenin (8;20;25;33;43). IKK recognizes and phosphorylates IκBα at a DSGIHS consensus sequence in the N-terminal region including serines 32 and 36. This consensus sequence is also found in the N-terminal of β-catenin at serines 33 and 37 (29).

The IKK complex is composed of a number of different proteins including the active catalytic components IKKα and IKKβ and the scaffold protein IKKγ/NEMO (34;44) (15). Several different stimuli including tumor necrosis factor α (TNFα) and interleukin 1 (IL-1) can stimulate the activity of the IKK complex. A number of kinases including MEKK1, NFκB inducing kinase (NIK), and atypical PKCs can activate IKK (17;22;26;27). Mechanical stress acting through integrins can also activate IKK (2). Once activated, the IKK complex phosphorylates IκB proteins, which lead to their degradation resulting in the release of the NF-κB proteins and their migration to the nucleus where they activate transcription of certain cellular genes. The NF-κB proteins are known to play a role in inflammatory responses as well as in apoptosis, cell survival, and development (reviewed in (17)).

IKKβ has higher activity for the IκB proteins and has a more significant role in the NF-κB pathway in response to activation such as TNFα and IL-1 than does IKKα (6;14;21;22;24;40). For example, IKKβ knockout mice die around embryonic day 12.5 from severe liver apoptosis, a phenotype that is similar to knockouts of the NF-κB component p65 and GSK3β. Fibroblasts taken from these embryos demonstrate decreased NF-κB activity and increased apoptosis due to unopposed TNFα stimulation. IKKα cannot compensate for the loss of IKKβ indicating that IKKβ is absolutely necessary for the regulation of this pathway. Knockouts of the IKKγ/NEMO gene which is critical for the control of IKKβ activity also result in severe hepatic apoptosis and defects in NF-κB activation in response to TNFα

and IL-1 treatment (35). In contrast, IKKα knockouts die perinatally with skeletal abnormalities including fused vertebrae, syndactyly, and missing phalanges. Upon TNFα and IL-1 stimulation, IKKα-/- cells exhibit somewhat reduced IκBα degradation and NFκB activity but the defect is not as severe as seen with disruption of the IKKβ gene. Moreover, these mice do not exhibit the severe hepatic apoptosis that characterizes the IKKβ, IKKγ/NEMO, and and GSK-3β knockout mice. These results demonstrate different roles for IKKα and IKKβ. In addition, IKKα knockout mice exhibit an unusual 10-fold thickening of the epidermis due to hyperproliferation of basal keratinocyte stem cells. Significantly, increased β-catenin levels are also associated with uncontrolled proliferation of epidermal basal cells and there are examples of wnt and LEF-1 involvement in hair and epidermal differentiation (10;45).

In the past 12 months we investigated the ability of IKK to regulate β-catenin signaling activity. We found that IKK exists in a complex with β-catenin and that expression of either IKKα or IKKβ can decrease β-catenin signaling. However, only a dominant negative (DN) IKKα mutant increased β-catenin signaling as well as protein levels. In addition, DN IKKα, but not DN IKKβ, inhibited the ability of APC to decrease β-catenin signaling. These results suggest that the IKK proteins are involved in β-catenin regulation.

Materials and Methods:

Cell culture and Transfections: SW480 colon cancer cells, SKBR3 breast cancer cells, and Cos-7 kidney cells were grown in DMEM supplemented with 5% fetal bovine serum. SW480 cells were transfected using lipofectamine plus (GibcoBRL). SKBR3 and Cos-7 cells were transfected using calcium phosphate (Promega). F. Mercurio kindly provided the IKKα (S176/180E and A) and IKK β (S177/181E and A) mutants (25). B. Vogelstein kindly provided APC. R. Pestell kindly provided the cyclin D1-luciferase promoter. Tularik Inc kindly provided the IKKα (K44M) and IKKβ (K44A) **(ref?)**.

Tcf/LEF Reporter Assay: Cells were plated at ~100,000 cells/well in a 12 well plate. After 24 hours, cells were transfected with the indicated DNA as well as TopFlash, which is a luciferase reporter containing Tcf/LEF response elements, or FopFlash, which is a Tcf/LEF mutated luciferase reporter, (described in (42)) and Renilla luciferase. All transfections were done in triplicate and repeated at least three times with the Tcf/LEF reporter activity measured in lumens after 48 hours using the luciferase assay (Promega). Dose responses were also performed and optimal doses chosen for these experiments.

Western Blotting: Cells were grown to confluence in 100 mm dishes (48 hours after transfection), washed twice with phosphate buffered saline (PBS), and lysed for 10 minutes on ice with NP-40 lysis buffer containing 1% NP-40 and protease inhibitors (1mM sodium vanadate, 50 mM sodium fluoride, and Boehringer Mannheim complete mini EDTA-free protease inhibitor cocktail). Lysates were centrifuged at 12,000 rpm at 4°C for 10 minutes. Protein content was measured by the BCA protein assay (Pierce). Western blotting was performed as previously described using β-catenin monoclonal antibody (Transduction Laboratories) at a 1:1000 dilution in 5% milk (38). Monoclonal HA antibody (Boerhinger Mannheim) was used at a concentration of 1 μg/ml, monoclonal FLAG antibody (Eastman Kodak) was used at a 1:500 dilution, and myc antibody was used at a concentration of 1 μg/ml. The blots were developed using chemiluminescent detection (Pierce).

Immunocytochemistry: Cells were plated on 18mm coverslips in 12 well plates at ~ 50,000 cells/well. Cells were transfected as above and fixed 48 hours later in 2% paraformaldehyde and permeabilized with 0.2% Triton. Monoclonal HA antibody (Babco) was used at a 1:1000 dilution and monoclonal FLAG antibody was used at a 1:500 dilution. Polyclonal β-catenin antibody (kindly provided by David Rimm)

was used at a 1:2000 dilution. All primary antibodies were incubated for 1 hour at room temperature. Fluorescein and Texas Red conjugated secondary antibodies (Kirkegaard and Perry Laboratories) were used at a 1:100 dilution and incubated for 1 hour at room temperature. Imaging was done on an Olympus Fluoview Confocal Laser Scanning Microscope.

Fractionation of Cellular Extracts: Cytoplasmic extracts were prepared according to Li et al (23) with slight modifications. Cos-7 cells were plated in 100 mM dishes (10^7 cells) and co-transfected with Flag-tagged IKKα and HA-tagged β-catenin. Prior to harvest, cells were washed twice with cold phosphate-buffered saline (PBS). Cells were next resuspended in buffer A (10 mM Hepes [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM EDTA) supplemented with phosphatase inhibitors (10 mM NaF, 10 mM β-glycerolphosphate, 0.5 μM okadaic acid, 1 mM sodium orthovanadate) and protease inhibitors (Roche Molecular Biochemicals). After incubation on ice for 10 min, cells were lysed in Wheaton all-glass Dounce homogenizer (Tight pestle). Nuclei were pelleted by centrifugation for 5 min at 2000 rpm (Beckman bench-top centrifuge, CH3.7 rotor). The supernatants were collected and mixed with 0.11 volume of buffer B (0.3 M Hepes [pH 7.9], 30 mM $MgCl_2$, and 1.4 M NaCl) and then centrifuged at 100,000 xg for 60 min at 4°C. The supernatants after ultra-centrifugation were termed S100.

Gel Filtration Chromatography: S100 were further fractionated on a Superdex-200 gel filtration column (Amersham Pharmacia Biotech) equilibrated with buffer D (20 mM Hepes [pH 7.9], 0.1 M KCl, 0.5 mM PMSF, 0.5 mM dithiothreitol, 0.2 mM EDTA, 20% glycerol). Protein markers (Sigma) used for the calibration of the column include bovine thyroglobulin (669 kDa), horse spleen apoferritin (443 kDa), b-amylase (200 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.5 kDa).

Protein Association Studies and Western Blot Analysis: For protein association studies, equal volumes from each of the Superdex-200 fraction were incubated overnight at 4 °C with 1 μg of indicated antibodies or normal mouse IgG. Immune complexes were collected with protein G-agarose (Sigma) for 2-3 h at 4 °C. Immunoprecipitates were analyzed by Western blotting using a chemiluminiscence system (ECL-Amersham Pharmacia Biotech).

Results:

To determine whether IKK could regulate β-catenin signaling we first transfected SW480 colon cancer cells with the constitutively active IKK mutants and a reporter construct, which reflects β-catenin activity. These constitutively active mutants (CA IKKα and CA IKKβ (SS EE)) were generated by changing serine residues located within the highly conserved activation loop at position 176 and 180 in IKKα and 177 and 181 in IKKβ, which mimic the phosphorylation induced conformational change resulting in kinase activation. SW480 cells contain a truncated APC gene and as a result have very high β-catenin levels and signaling. To measure β-catenin signaling, we used a luciferase promoter containing Tcf/LEF response elements (TopFlash) and measured the lumens generated. A Tcf/LEF mutated luciferase reporter (FopFlash) was used as a control. β-catenin signaling decreased by 80-90% upon co-transfection of either CA IKKα or CA IKK β expression, (Figure 1A). In contrast, CA IKKα increased NF-κB reporter activity in the same experiment. To further investigate the requirement for IKK kinase activity, we next transfected kinase dead IKK α and β mutants, in which a conserved lysine in the ATP binding site at position 44 was mutated to either methionine in IKKα or alanine in IKKβ, into SW480 cells. Neither the kinase dead IKKα nor IKKβ mutant decreased β-catenin activity indicating that intact

kinase function for both IKK α and β is necessary for their effects on β-catenin signaling (Figure 1B). These data indicate that IKKα and IKKβ are capable of regulating β-catenin signaling.

To more clearly address the role of these kinases, we transfected either dominant negative (DN) IKKα (S176/180A) or IKKβ (S177/181A) into SKBR3 breast cancer cells (Figure 1C). The alanine substitutions for serine residues in the activation loops of these kinases prevent kinase activation in response to TNFα and IL-1 stimulation. SKBR3 cells have very low levels of β-catenin and consequently the low levels of β-catenin activity permit easier detection of changes in its signaling activity. The DN IKKα increased β-catenin signaling over 5 fold and significantly increased β-catenin protein levels (Figure 1C). In contrast, the DN IKKβ had no effect on β-catenin signaling or protein levels. Neither CA IKKα nor CA IKKβ affected β-catenin signaling or protein levels in SKBR3 cells. These results indicate that blockade of endogenous IKKα activation but not of IKKβ activation has a direct effect on the activity of the IKK complex toward β-catenin in SKBR3 cells. This result contrasts with failure of DN IKKα to block IKK activity toward IκBα. We next investigated the ability of DN IKKα to regulate the phosphorylation-dependent molecular weight shift observed in SKBR3 cells when β-catenin is over-expressed (Figure 1D). Consistent with a role for IKKα in regulating β-catenin phosphorylation the higher molecular weight form of β-catenin was absent in cells co-expressing DN IKKα. Western blot analysis was then performed to determine the levels of endogenous IKKα and β in the different cell lines used in these studies. We found SKBR3, SW480, and Cos-7 cells to have significantly more IKKα than IKKβ using the indicated IKKα and IKKβ antibodies while Jurkat cells, which were used as a positive control, expressed similar levels of IKKα and β (Figure 1E).

To determine whether IKKα influences the cellular localization of β-catenin, immunocytochemistry was performed using SKBR3 cells transfected with either HA-tagged DN IKKα or FLAG-tagged DN IKKβ. Cells were stained for either IKKα or IKKβ using antibodies directed against either the HA or FLAG tag as well as β-catenin. DN IKKα was found in both the nucleus and the cytoplasm in the majority of transfected cells (Figure 2 A1). β-catenin levels were significantly increased in DN IKKα transfected cells and in most cases β-catenin co-localized with DN IKKα in the nucleus (Figure 2 A2). In cells transfected with DN IKKβ, IKKβ was found only in the cytoplasm (Figure 2 B1) and there was no change in β-catenin levels or localization (Figure 2 B2). CA IKKα also localized to the nucleus (not shown).

To demonstrate whether IKK could regulate β-catenin activated transcription of a known target gene, a cyclin D1-luciferase promoter was transfected into SW480 cells. This cyclin D1 promoter construct (-163CD1Luc) contains Tcf/LEF sites as well as CREB, AP1, SP1, and NF-κB sites (31;37). As was the case with the TCF-reporter, TopFlash, both the constitutively active IKKα and IKKβ proteins decreased cyclin D1 promoter activity in SW480 cells (Figure 3). In contrast, a cyclin D1 promoter with mutated Tcf/LEF sites, but retaining the other regulatory elements, was not responsive to either IKKα or IKKβ. Because NFκB has been shown to positively regulate cyclin D1 promoter activity these data indicate that, in APC-mutant colon cancer cells, the activity of the -163 cyclin D1 promoter is predominantly regulated by IKK control of β-catenin not NF-κB signaling.

When wild type (WT) APC is over-expressed in SW480 cells, β-catenin signaling is dramatically reduced. In earlier studies, we found that GSK3β activity was not required for APC to decrease β-catenin signaling (7). To determine if IKK activity was required for APC to regulate β-catenin activity, SW480 cells were co-transfected with APC and DN IKKα or DN IKKβ (Figure 4). Remarkably, DN IKKα but

not DN IKKβ completely inhibited the ability of APC to decrease β-catenin signaling. These results suggest that IKKα can directly alter β-catenin signaling perhaps by association with the IKK complex.

Finally, to determine whether IKKα could directly interact with β-catenin, immunoprecipitation was performed in Cos-7 kidney cells co-transfected with HA-tagged β-catenin and FLAG-tagged IKKα. Before immunoprecipitation cytoplasmic cell extracts were fractionated over a sizing column. Figure 5 shows that β-catenin is present in IKKα immunoprecipitates and that IKKα is also present in β-catenin immunoprecipitates. Thus IKKα can directly associate with a protein complex, which includes β-catenin.

Several studies point to separate roles for IKKα and IKKβ in regulating the activity of the IKK complex (reviewed in (16)). IKKβ activity is essential for the ability of the IKK complex to regulate IκB protein stability and thus control the transcription of NF-κB regulated genes. Although both exogenously expressed and endogenous IKKα can regulate NF-κB activity, it does not appear to have a dominant role in activating the NF-κB pathway. These results suggest that IKKα likely has targets other than the IκB proteins. In the present study we show that the IKK complex can also regulate β-catenin protein levels and signaling activity. This is the first demonstration of an IKK substrate other than the IκB proteins.

In contrast to the dominant role of IKKβ in NFκB signaling, our experiments show that IKKα is the dominant IKK component involved in β-catenin regulation. When over-expressed, both constitutively active IKKα and IKKβ can decrease β-catenin signaling in SW480 cells, which have high endogenous levels of β-catenin. However, these kinases have no detectable effect in SKBR3 cells, which have low endogenous levels of β-catenin. Only the dominant negative IKKα, but not IKKβ, can increase β-catenin protein levels and signaling in SKBR3 cells. Furthermore, only the dominant negative IKKα, but not IKKβ, can reverse the inhibitory effects of APC on β-catenin signaling in SW480 cells. Taken together these data suggest a model in which signals that activate the IKK complex through IKKα can target a complex containing β-catenin whereas signals that activate the IKK complex through IKKβ target it for IκBα. Alternatively, it is possible that a subcomplex of IKKα homodimers can bind to β-catenin. A role for IKKα in the regulation of β-catenin signaling in epithelial cells is consistent with it potential role in the described phenotype of the IKKα -/- mouse and of epidermal cells over-expressing β-catenin. Each of these phenotypes is characterized by hyperproliferation of basal stem cells (14;21;40;45). A role for the IKKα/β-catenin/TCF pathway in the regulation of epithelial stem cell proliferation is also consistent with the phenotype of the TCF4 -/- mouse which completely lacks the stem cell compartment of the intestine (9).

The precise mechanism by which IKK regulates β-catenin is not clear but is likely to involve phosphorylation of one or more of the N-terminal serine and threonine residues known to regulate the stability and perhaps nuclear localization of both proteins (8;43). Like β-catenin, IκBα has a function in both the cytoplasm and the nucleus (1). In addition to its role as a cytoplasmic inhibitor of NF-κB nuclear translocation, nuclear IκBα can also directly inhibit NF-κB mediated transcriptional activation by preventing its DNA binding properties. Similarly, cytoplasmic β-catenin is involved in cell-cell adhesion and in the indirect regulation of PKA-regulated genes (?) in addition to its role as a nuclear co-activator of TCF/LEF regulated transcription (4;19). Remarkably, both IκBα and β-catenin can also interact with and regulate the function of retinoid receptors. IκBα can negatively regulate RXR-mediated transcription whereas β-catenin can act as a co-activator of RAR-mediated transcription. Both β-catenin and NF-κB can bind to the coactivator protein CBP. Our results show that both IKKα and IKKβ are present in the cytoplasm but that only IKKα is able to localize to the nucleus. This differential localization of the two IKK kinases may be relevant for the precise regulation of both nuclear and cytoplasmic functions of IκBα and β-catenin. For example it is possible that IKKα could have two roles in the regulation of β-catenin

signaling. IKKα could phosphorylate β-catenin and target it for ubiquitination and could also directly interact with it to prevent its co-activator function in the nucleus. Perhaps IKKβ can also regulate β-catenin phosphorylation in the cytoplasm but lacks the nuclear activity of IKKα.

Although APC is most commonly associated with wnt signaling, genetic studies differ on its role in this pathway including whether it antagonizes or potentiates wnt signaling (36). Similarly, although genetic studies show that GSK-3β is a component of the wnt pathway and that inhibition of GSK-3β activity elevates β-catenin signaling and proteins levels in several different in vitro systems, GSK-3β activity is not required for APC to regulate β-catenin signaling or protein levels (7). However the present study shows that IKKα (but not IKKβ) is required for APC-mediated inhibition of β-catenin signaling. Other data shows that the phosphorylation event that results in the formation of the slower migrating form of β-catenin only occurs when β-catenin is overexpressed in cells expressing wild-type APC (Fig. 1D). The generation of this slower migrating species is completely blocked by co-expression of DN IKKα. These data indicate that elevation of β-catenin protein levels, by wnt signaling for example, results in the increased activity of a kinase (IKKα?), which in turn phosphorylates the accumulated cytoplasmic and nuclear β-catenin to target it for degradation (Figure 6). In this model the IKK complex could be involved in the feedback regulation of pathways, such as wnt signaling, that result in elevated β-catenin levels. IKKα protein levels are similar in cells that express very low levels of β-catenin (SKBR3 cells) and in cells that express extremely high levels of β-catenin (SW480) and indicates that β-catenin does not regulate IKKα protein levels. Consequently, we favor a model in which elevated β-catenin protein levels directly trigger activation of the IKK complex. Perhaps the role of APC is to facilitate the β-catenin-mediated activation of the IKK complex and complete the negative feedback loop. Our demonstration that constitutively active IKK can decrease β-catenin signaling independently of APC is consistent with this model. β-catenin can also regulate the levels of the ubiquitin ligase component βTRCP which can in turn increase IκBα degradation and subsequent NFκB activation as well as negatively regulating its own activity (39). Taken together with the recent demonstration that GSK-3 is involved in the regulation of NFκB activity, these data indicate that there is a significant amount of "cross-talk" between these two pathways (3;13). Our results strongly suggest that substrate specificity of the IKK complex is regulated by the relative contribution of IKKα and IKKβ. Determining the precise mechanism by which IKK regulates β-catenin signaling as well as the factors specifying activity will further elucidate the relationship between these two important pathways.

Figure Legends

Figure 1: IKKα regulates β-catenin signaling and protein levels.
A luciferase reporter containing Tcf/LEF response elements (TopFlash) was used to detect the effects of IKK on β-catenin signaling. All transfections were normalized with equal amounts of DNA and performed in triplicate. Data is measured in lumens and plotted as percent control. A) SW480 cells were transfected using lipofectamine plus with PCDNA3-cat control DNA and/or 0.1 μg constitutively active (CA) IKK α (S176/180E) or IKK β (S177/81) as well as 0.1 μg of TopFlash and 0.002 μg of Renilla luciferase, which was transfected as a control for transfection efficiency. Both CA IKKα and CA IKK β decreased β-catenin signaling by 80-90%. B) SW480 cells were transfected with 0.2 μg of kinase dead IKKα (K44M) or IKKβ (K44A), 0.1 μg of TopFlash, and 0.002 μg of Renilla. Kinase dead IKKα and β had no effect on β-catenin signaling indicating that IKK kinase activity is necessary. C) SKBR3 cells were transfected with 1 μg of dominant negative (DN) IKKα (176/190A) or IKK β (S177/181A) as well as 1 μg of TopFlash, and 0.02 μg Renilla. DN IKKα increased β-catenin signaling by over 5 fold whereas DN IKKβ had no effect. FopFlash, which is a Tcf/LEF mutated luciferase reporter, was used as a control and was unaffected by IKK. D) SKBR3 cells were transfected with 10 μg DN IKKα or β. NP-40 lysates were probed using a monoclonal antibody to β-catenin. DN IKKα significantly increased β-catenin protein levels whereas DN IKKβ had no effect. SKBR3 cells were also co-transfected with 10 μg of wild-type (WT) β-catenin and DN IKKα and probed for β-catenin expression. Overexpression of WT β-catenin generates a phosphorylated form of β-catenin and two bands (arrows). Co-expression of DN IKKα prevents this phosphorylated form of β-catenin. E) NP-40 lysates of cell lines used in this study were probed using monoclonal antibodies to IKKα and β. These cells have significantly more IKKα than IKKβ indicating that IKKα has a more prominent role. Jurkat cell lysates were used as a control.

Figure 2: DN IKKα increases nuclear β-catenin levels.
Immunocytochemistry was performed to determine the effect of DN IKKα on β-catenin localization in SKBR3 cells. Cells were transfected with 1 μg of HA-tagged DN IKKα for 48 hours, fixed in 2% paraformaldehyde and 0.2% Triton, and stained with monoclonal HA antibody (A1). DN IKKα was found in both the cytoplasm and nucleus in the majority of transfected cells. These cells were also double-stained for endogenous β-catenin using a polyclonal β-catenin antibody (A2). β-catenin levels were significantly higher in transfected cells and in most cases co-localized with DN IKKα in the nucleus. In SKBR3 cells transfected with 1 μg of FLAG-tagged DN IKKβ, DN IKKβ was found predominantly in the cytoplasm (B1). Increased β-catenin protein levels were not observed nor was β-catenin detected in the nucleus.

Figure 3: IKKα regulates cyclin D1 promoter activity.
To determine if IKK could regulate the expression of a β-catenin target gene, a cyclin D1-luciferase promoter (0.5 μg) was used. Both CA IKKα and CA IKKβ decreased cyclin D1 promoter activity in SW480 cells. (Note that data is reported as arbitrary units.) A cyclin D1 promoter with mutated Tcf/LEF sites (ΔLEF) was used as a control.

Figure 4: DN IKKα inhibits the ability of APC and APC2 to decrease β-catenin signaling.
To determine the effects of IKKα on APC activity, SW480 cells were co-transfected with 0.1 μg APC and 0.1 μg DN IKKα or β in addition to TopFlash and Renilla. APC decreases β-catenin TopFlash

signaling by 80 %. (Data is reported as percent control.) DN IKKα but not DN IKKβ completely inhibited the ability of APC to decrease β-catenin signaling.

Figure 5: IKKα interacts with β-catenin.
Superdex-200 column fractions derived from S100 Cos cells trasfected with Flag-tagged IKKα and Ha-tagged β-catenin were immunoprecipitated with mouse IgG (lane 1 and 3) or anti-Flag (lane 2) or anti-HA antibody (lane 4). The immunoprecipitates were subjected to fractionation by SDS PAGE, and immunoblotted with antibodies to HA (lanes 1 and 2) or Flag (lanes 3 and 4). β-catenin associates with IKKα.

Figure 6: Potential model for IKKα regulation of β-catenin.
Wnt signaling increases β-catenin protein levels by inactivating GSK-3. We have shown that IKKα directly regulates β-catenin protein levels and signaling activity. This suggests that elevation of β-catenin leads to IKKα activation and subsequent β-catenin phosphorylation and degradation creating a feedback loop. It is possible that β-catenin regulates transcription of an IKK kinase, such as NIK, thus activating IKK and down-regulating itself. We also showed that IKKα activation is necessary for APC regulated inhibition of β-catenin signaling. APC may be necessary to facilitate β-catenin targeting by IKKα. In addition, βTRCP is upregulated by β-catenin, consequently decreasing β-catenin levels and down-regulating its own activity as well as increasing NF-κB activity. These data indicate a significant amount of "cross-talk" between these two pathways.

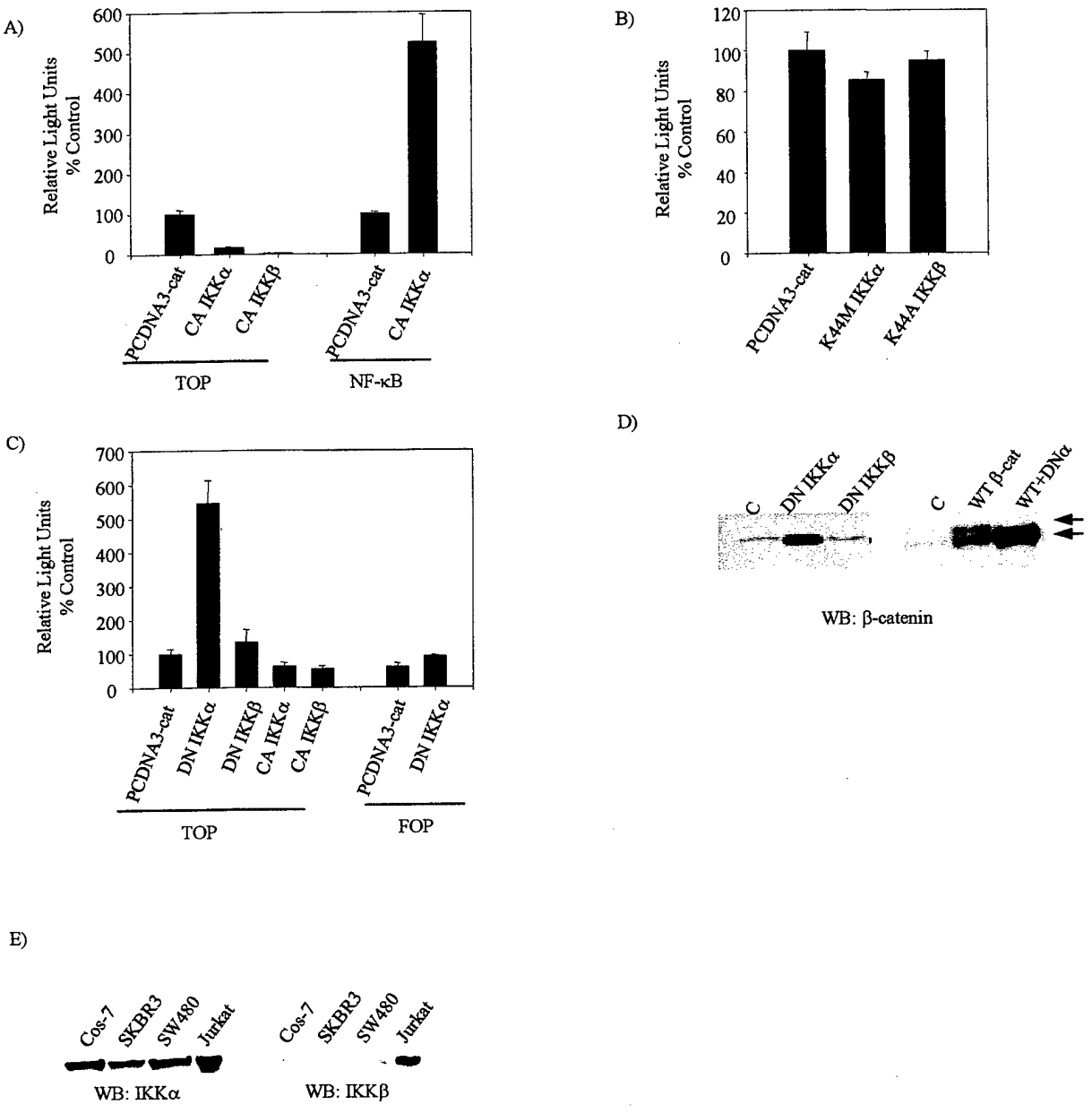
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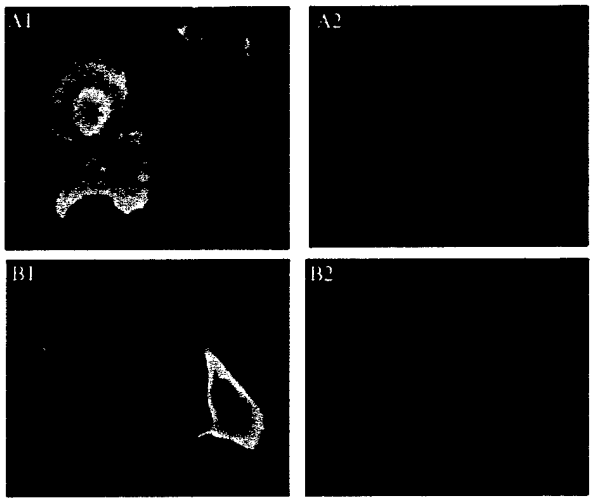
Figure 1



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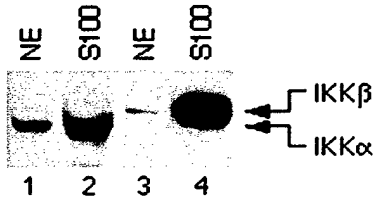

Figure 2

A)

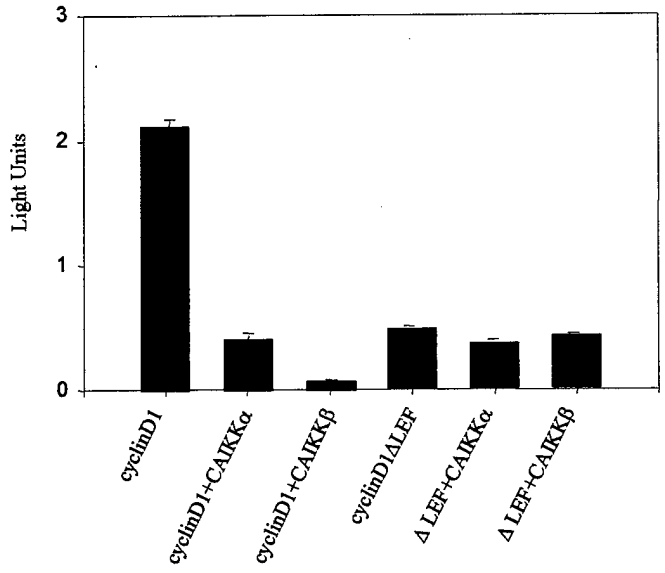
Light Units
3
2
1
0
cyclinD1
cyclinD1+CAIKKα
cyclinD1+CAIKKβ
cyclinD1ΔLEF
ΔLEF+CAIKKα
ΔLEF+CAIKKβ

Figure 3

A)

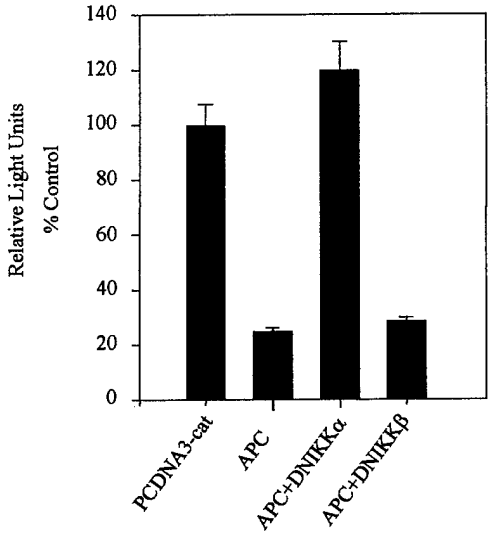
Relative Light Units
% Control
140
120
100
80
60
40
20
0
PCDNA3-cat
APC
APC+DNIKKα
APC+DNIKKβ

Figure 4

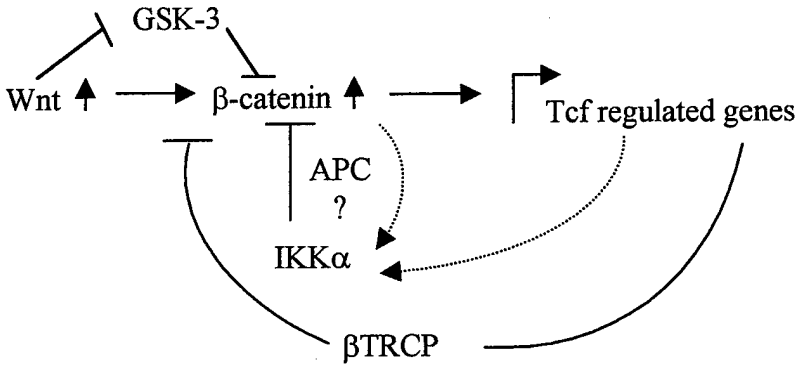

Figure 6

Key Research Accomplishments:

1) Identification of the IKK complex as a β-catenin-directed kinase
2) Demonstration that IKKα, not IKKβ is the active kinase
3) Demonstration that the activity of the tumor suppressor APC requires IKKα, but not IKKβ

Reportable Outcomes: The IKK complex regulates β-catenin signaling. Paper in preparation

Conclusions: The wnt/β-catenin and NFκB pathways regulate the transcription of genes that are involved in cell cycle control and cellular differentiation. In addition the NF-κB pathway is involved in the induction of the inflammatory response. β-catenin, a known oncogene, is an important component of the wnt signaling pathway and IκBα is an important regulator of the NF-κB pathway. Both proteins are phosphorylated at serines in the N-terminal region, which subsequently target them for ubiquitination by the same ubiquitin ligase complex. The kinases that are important in the phosphorylation of these proteins have been intensely studied. The IKK complex is responsible for the phosphorylation of IκBα while GSK-3β is thought to regulate β-catenin phosphorylation. The IKK complex contains two kinases, IKKα and IKKβ. Gene disruption studies in mice indicate that IKKβ is the dominant component of the IKK complex involved in phosphorylation of IκBα. We now show that IKK also exists in a complex with β-catenin and that expression of either IKKα or IKKβ can decrease β-catenin signaling in APC-mutant colon cancer cells with high endogenous β-catenin levels. However, only a dominant negative (DN) IKKα mutant increased β-catenin signaling and protein levels in cells with low endogenous β-catenin. In addition, DN IKKα, but not DN IKKβ, completely inhibited the ability of APC to decrease β-catenin signaling in colon cancer cells. These results indicate that, in contrast to IKK control of NFκB signaling, IKKα not IKKβ has the dominant role in the regulation of β-catenin activity.

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